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Detection of K-ras and p53 mutations in sputum samples of lung cancer patients using laser capture microdissection microscope and mutation analysis

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Abstract

Mutations in the p53 tumor suppressor gene and the K-ras oncogene have been frequently found in sputum and bronchoalveolar lavage (BAL) samples of lung cancer patients and other patients prior to presenting clinical symptoms of lung cancer, suggesting that they may provide useful biomarkers for early lung cancer diagnosis. However, the detection of these gene mutations in sputum and BAL samples has been complicated by the fact that they often occur in only a small fraction of epithelial cells among sputum cells and, in the case of p53 gene, at many codons. In this study, sputum cells were collected on a filter membrane by sputum cytocentrifugation and morphologically analyzed. Epithelial cells were selectively taken by using a laser capture microdissection microscope and analyzed by polymerase chain reaction (PCR) and single-stranded conformational polymorphism (SSCP) for p53 mutations and by PCR and denaturing gradient gel electrophoresis (DGGE) for K-ras mutations. This method was used to analyze sputum of 15 Chinese women with lung cancer from Xuan Wei County, China and detected mutations in sputum of 7 (46.7%) patients, including 5 patients with p53 mutations, 1 patient with a K-ras mutation, and 1 patient with K-ras and p53 mutations. For comparison, only two of the mutations were detected by conventional methods. Therefore, the laser capture/mutation analysis method is sensitive and facilitates the detection of low-fraction mutations occurring throughout the p53 and K-ras genes in sputum of lung cancer patients. This method may be applicable to the analysis of epithelial cells from clinically normal sputum or BAL samples from individuals with a high risk for developing lung cancer.

Keywords: Lung cancer; Sputum; Laser capture microdissection; K-ras and p53 mutations

Lung cancer remains the most common cause of death from cancer worldwide [1]. In the United States, this disease kills both men and women more than any other type of cancers, causing an estimated 156,900 deaths in 2000 despite improved therapy and a nationwide antismoking campaign [2]. One goal of cancer researchers has been to develop assays that facilitate early detection and treatment of lung cancer and thus decrease the mortality of this disease [3–5].

Lung cancer, like other cancers, results from the accumulation of genetic alterations in genes involved in the control of cell growth and differentiation [6,7]. Mutations in two of these genes, the K-ras oncogene and the p53 tumor suppressor gene, have been frequently found in lung tumors and are implicated in the development of lung cancer. K-ras mutations occur in 20 to 50% of adenocarcinoma and undifferentiated large-cell carcinoma of the lung and, to a lesser extent, in squamous cell carcinoma [8–12]. More than 90% of K-ras mutations were detected at codon 12 of the K-ras gene [12]. Therefore, these mutations can be easily detected using sufficiently sensitive methods. The p53

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mutations occur at a frequency of 30 to 50% in lung tumors and cell lines [13–19]. Contrarily to K-ras mutations, p53 mutations occur at more than 100 sites of the p53 gene. In lung cancer, a high fraction of these mutations are found clustered as "hot spots" at codons 158, 175, 245, 248, 249, 273, and 282 within exons 5 to 8 where the four evolutionarily conserved domains of the p53 gene are located [20–23]. The high prevalence of mutations in both the K-ras and the p53 genes in lung cancer should make these mutations useful biomarkers for lung cancer.

To evaluate the roles of K-ras and p53 mutations in lung carcinogenesis and establish their significance as early detection biomarkers, such as their detection in sputum and bronchoalveolar lavage (BAL),[1] one needs sufficiently sensitive methods to determine a complete spectrum of mutations in these genes in these specimens. Several molecular approaches have been applied to enhance the detection of point mutations in cultured cells, tissues, or sputum samples, including mismatch amplification mutation assay [24], polymerase chain reaction (PCR) and restriction length polymorphism [25–28], enriched PCR [29–31], PCR-based cloning and specific probe hybridization [32], and ligase chain reaction [33]. Another specific and sensitive method has been developed for K-ras mutation detection by improvement upon existing methodology. Through a combination of PCR, mutant allele enrichment (MAE), nested amplification, and denaturing gradient gel electrophoresis (DGGE), a sensitivity of detection of 1 mutated cell in 10^4 to 10^5 normal cells [34] can be attained. This method has been previously applied to analyze K-ras codon 12 mutations in sputum samples of lung cancer patients [35]. Nevertheless, all these methods are usually applicable to detect mutations at specific codons of the genes investigated. In the case of the p53 tumor suppressor gene in lung cancer, more than half of the mutations occur outside the hotspot codons and are thus not detectable by most existing sensitive methods that target mutations at specific codons.

The aim of this study was to find means to detect a complete spectrum of low-fraction p53 and K-ras mutations in sputum samples of lung cancer patients. We combined sputum cytocentrifugation with a laser capture microdissection microscope to isolate epithelial cells from sputum samples. We then screened for K-ras and p53 mutations in these isolated cells using PCR and DGGE or single-stranded conformational polymorphism (SSCP). We used this approach to analyze sputum samples obtained from 15 Chinese women with lung cancer from Xuan Wei County, China, where residents were exposed to smoky coal combustion emissions in unvented homes and the mortality rate for lung cancer in nonsmoking women is eight times that of the Chinese national average lung cancer rate [36]. We compared this mutation detection method with existing sensitive methods that used repeated steps of mutant allele enrichment from DNA extracted directly from sputum samples before analysis of mutant sequences by DGGE or SSCP.

[1] *Abbreviations used:* BAL, bronchoalveolar lavage; MAE, mutant allele enrichment; DGGE, denaturing gradient gel electrophoresis; SSCP, single-stranded conformational polymorphism.

Materials and methods

Lung cancer patients and sputum samples

Xuan Wei patients with lung cancer who donated sputum samples used in this study were a subset of the patients who were initially involved in a previous study [36]. These patients were examined for their clinical symptoms and by chest X-ray analysis at the Xuan Wei Hospital and confirmed by results of histological analysis. Sputum samples were obtained from a total of 15 patients identified at Xuan Wei Hospital. For these patients, a standardized closed questionnaire was used to obtain demographic information, smoking history, family and personal medical history, and information on other variables [36]. For the protection of human subjects, this study was conducted according to recommendations of the World Medical Association Declaration of Helsinki (1989) [37]. The study subjects provided informed consent to participate in this study. The research protocol met the requirements for protection of human subject certification by the US EPA. The collection of sputum and preparation of sputum cells were described previously [36]. The cells were subjected to cytological examination using the method described by Saccomanno to determine whether the sputum samples were derived from the lower respiratory tracts and to confirm the presence of tumor and/or atypical cells in each sputum sample [36].

Strategy for mutation analysis

Each sputum sample was subjected to mutation analysis by three different methods. In the first two approaches, DNA was first extracted from each sputum sample. An aliquot of each DNA was then screened for mutations by PCR and DGGE (for K-ras) or SSCP (for p53), which can detect mutations present at a mutant fraction of at least 5–10% over a nonmutant background [34] (Method 1). Another aliquot of each DNA was analyzed by using more sensitive methods that included a step of MAE by two-rounds of PCR and restriction enzyme digestion at the codons investigated, followed by DGGE analysis (for K-ras) or polyacrylamide gel electrophoresis (PAGE) analysis (for p53) (Method 2). This codon-specific method allowed detection of mutations present at a mutant fraction of 10^{-4} in a nonmutant background [34]. The codons investigated in this study

included codon 12 of the K-ras gene, where more than 90% of all K-ras mutations were detected [12], and codons 158, 175, 245, 248, 249, 273 and 282, of the p53 gene, where almost half of all p53 mutations were detected in lung tumors [20–23]. In the final method, malignant and/or atypical cells were first isolated from the excess of other sputum cells, including mostly leucocytes and buccal epithelial cells. The isolated cells were then used for analysis of K-ras mutations and p53 mutations, using the non-codon specific PCR + DGGE and PCR + SSCP methods, respectively (Method 3).

Conditions for DNA extraction from sputum and mutation analysis using Methods 1 and 2

DNA extraction. For DNA extraction, half of each sputum sample in Saccomanno's fluid containing approximately 2.5×10^4 cells was centrifuged. The cell pellet was washed twice with phosphate-buffered saline, resuspended in a lysis buffer (10 mM Tris, pH 7.4, 0.5% SDS, 150 mM NaCl, 100 mM EDTA), and digested with RNase A1 (10 mg/ml, at 37 °C for 2 h) and proteinase K (20 μg/ml, at 37 °C for 4 h). DNA was recovered by phenol–chloroform extraction and ethanol precipitation. The DNA was resuspended in H_2O and kept at −20 °C.

K-ras mutation analysis in sputum DNA. K-ras mutations were analyzed by both PCR + DGGE (Method 1) and PCR + MAE + DGGE (Method 2) which can detect K-ras mutations present at a mutant fraction among a nonmutant background of 5×10^{-2} and 10^{-4}–10^{-5}, respectively [34].

p53 mutation analysis in sputum DNA. The analysis of mutations in exons 5–8 of the p53 gene by PCR + SSCP (Method 1) was carried out as described previously [38]. For a sensitive analysis of p53 mutations occurring at codons 158, 175/176, 245, 248, 249, 273, and 282, we used PCR + MAE + PAGE (Method 2). These codons were located within three exons, including exons 5, 7, and 8. To simplify the screening for mutations at each codon in each DNA sample, we performed DNA amplification of these three exons simultaneously in the same PCR. Each amplified DNA sample was then used as a template for a second round of amplification of each exon individually. The first PCR amplification was carried out for 12 cycles using three pairs of primers in the same PCR mixture, including exon 5 [pE5 sense: 5′-TTC CTC TTC CTA CAG TAC TC-3′; pE5 anti-sense: 5′-CGC TAT CTG AGC AGC GCC CA-3′], exon 7 [pE7 sense: 5′-GTA ACA GTT CCT GCA TGA GC-3′; pE7 anti-sense: 5′-TCT TCC AGT GTG ATG ATG GTG AGG ATA GG-3′], and exon 8 [pE8 sense: 5′-GAC GGA ACA GCT TTG AGG CG-3′; pE8 anti-sense: 5′-GGT GAG GCT CCC CTT TCT TG-3′]. The amplified fragments were then used as a stock template to amplify and analyze each p53 gene codon of interest individually, using the appropriate pair of primers above, as described below.

The first PCR was carried out in a 50-μl reaction mixture containing 10 mM Tris–HCl, pH 8.0, 2.5 mM $MgCl_2$, 50 mM KCl, 100 μM each dNTP, 0.25 μM each of the three pairs of the p53 gene primers, and genomic DNA. The amplification was carried out using 1 unit of Gold *Taq* DNA polymerase, first at 95 °C for 9 min and then at 94 °C/1 min, 65 °C/2 min, and 72 °C/2 min, for 12 cycles.

The stock template, obtained from the first round of amplification, contained (a) a p53 gene exon 5 segment of 196 bp with codons 158 and 175/176, which correspond to *Aci*I and *Nco*I restriction enzyme site, respectively, (b) a p53 gene exon 7 segment of 64 bp with hotspot codons 245, 248, and 249, which correspond to *Bsr*BI, *Msp*I, and *Stu*I sites, respectively, and (c) a p53 gene exon 8 segment of 93 bp with hotspot codons 273 and 282, which correspond to *Bst*UI and *Msp*I sites, respectively. To increase the fraction of mutant carrying each of the hotspot codons of interest, 1 μl of the first-round PCR stock template was diluted in a final 4 μl reaction mixture containing 1× appropriate buffer and 3 units of the appropriate enzyme (New England Biolab, MA), for 2 h at 37 °C (60 °C for *Bst*UI). The digestion mixture was heated at 85 °C for 5 min and then diluted to a final 25-μl PCR mixture (see the conditions above) containing the appropriate primers shown above and 0.25 μl [α-^{32}P]dATP (NEN, Boston, MA). The amplification was carried out using 2 units of Gold *Taq* DNA polymerase, for 30 cycles (94 °C/1 min, 60 °C/2 min, and 72 °C/2 min) after heating at 95 °C/9 min. Ten microliters was diluted into a final 50 μl volume with the appropriate buffer and digested with 20 units of each enzyme as described above. The digested material was ethanol precipitated and separated through a 10% gel. The remaining undigested fragment was purified from the gel and sequenced to determine the nature of mutation(s).

Method 3: laser capture/mutation analysis of epithelial cells isolated from sputum

Preparation of epithelial cells from sputum. Each sputum sample containing an equivalence of 5000 cells was centrifuged to remove the Saccomanno fluid. The cells were resuspended in 1 ml of phosphate saline buffer and transferred onto a Cyto-Tek specimen chamber. The chamber was fixed onto a holder equipped with a membrane filter and centrifuged using a Cyto-Tek centrifuge (VWR, Bridgeport, NJ). The filter was recovered from the chamber and air-dried. The cells retained on the filter were stained with eosine and hemotoxyline and histopathologically analyzed. Approximately 150 epithelial cells were captured on a "cap" using a laser capture microdissection microscope. Buccal cells were also taken from each sputum sample, analyzed, and compared with the matched epithelial cells.

DNA extraction and mutation analysis. The captured cells were lysed by adding directly on each cap 15 μl of lysis solution (40 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5% Tween–20, and 0.5 μg/μl proteinase K). The resulting cell lysate was recovered in the microcentrifuge tube by a quick and gentle spinning in a microcentrifuge and then heated at 95 °C for 5 min to inactivate the proteinase K [39].

For K-ras mutation analysis, lysis solution containing an equivalence of 20 epithelial cells was used for PCR amplification in a 25 μl reaction mixture and analyzed by DGGE, as described previously [12,34]. The isolated cells were also analyzed for p53 mutations using the PCR + SSCP method and the primers and reaction conditions described previously [38]. Briefly, lysis solution containing an equivalence of 20 cells was used for a first round of PCR amplification of three fragments of the p53 gene, containing exons 5, 6, 7, and 8 in a single reaction, for 12 cycles. Then 1 μl of each amplified product was used for a second round of amplification of each fragment separately in a 25-μl reaction mixture containing the reagents, appropriate pair of primers, and 0.25-μl [α-^{32}P]dATP (NEN). The amplification was carried out using 2 units of Gold *Taq* DNA polymerase, for 35 cycles after heating at 95 °C/9 min (94 °C/1 min, 60 °C/2 min, and 72 °C/2 min). One microliter of each reaction was analyzed by SSCP [38,40]. The gel was dried and autoradiographed. Mutant allele DNA was isolated from the gel and further characterized for mutations.

Results and discussion

We first analyzed DNA extracted from each sputum sample using PCR + DGGE for K-ras mutations and PCR + SSCP for mutations in exons 5 to 8 of the p53 gene. The results showed no K-ras or p53 mutation in DNA from any the 15 patients (data not shown). However, since these methods allow detection only of mutations representing at least 5–10% of a non-mutant background [12,34], any mutation present at a lower mutant fraction in these sputum samples would have not been detected. It was expected that in sputum of lung cancer patients K-ras or p53 mutations occurred in only a small proportion of epithelial cells which themselves represented a small fraction of exfoliated cells found in sputum. We thus applied more sensitive methods to analyze these mutations.

A fraction of each DNA sample above was further analyzed by using the codon-specific but more sensitive PCR + MAE + DGGE for K-ras mutations and PCR + MAE + PAGE for p53 mutations. As shown in Table 1, two mutations (13.3%) were identified among 2 of the 15 patients, including a GGT to TGT mutation at codon 12 of the K-ras gene (patient 4) and a CGG to CAG at codon 248 of the p53 gene (patient 13). Therefore, K-ras and p53 mutations were present in sputum samples of lung cancer patients from Xuan Wei County, China and could be detected by only using sensitive methods targeting mutations at specific codons of the K-ras and p53 genes. Furthermore, it was anticipated that additional mutations occurring outside the codons investigated in some of the sputum samples were not detected by these codon-specific methods. However, it would be impractical to screen for mutations in many more codons to identify most of the mutations that might occur in the p53 and K-ras genes in sputum of these patients.

To overcome these limitations, we first isolated epithelial cells from each sputum sample by sputum

Table 1
Summary of p53 and K-ras mutations of 15 lung cancer patients from XuanWei, China

Patient	p53		K-ras	
	Mutations	Amino acid changes	Mutations	Amino acid changes
1				
2[a]	E5cod.136 CAA to CAG	Gln to Gln		
3				
4[b]			Cod.12 GGT to TGT	Gly to Cys
5				
6				
7[a]	E5cod.151 CCC to TCC	Pro to Ser		
8[a]	E7cod.244 GGC to TGC	Gly to Cys		
9				
10[a]	E5cod.139 AAG to TAG	Lys to Stop	Cod.13 GGC to TGC	Gly to Cys
11				
12				
13[b]	E7cod.248 CGG to CAG	Arg to Gln		
14[a]	E7cod.244 GGC to GTC	Gly to Val		
15				

[a] Mutations detected only in tumor cells isolated from sputum by the laser capture/mutation analysis method.
[b] Mutations detected both by the PCR + MAE + PAGE (for p53) or PCR + MAE + DGGE (for K-ras) method in DNA extracted from sputum cells and by laser capture/mutation analysis method in tumor cells isolated from sputum.

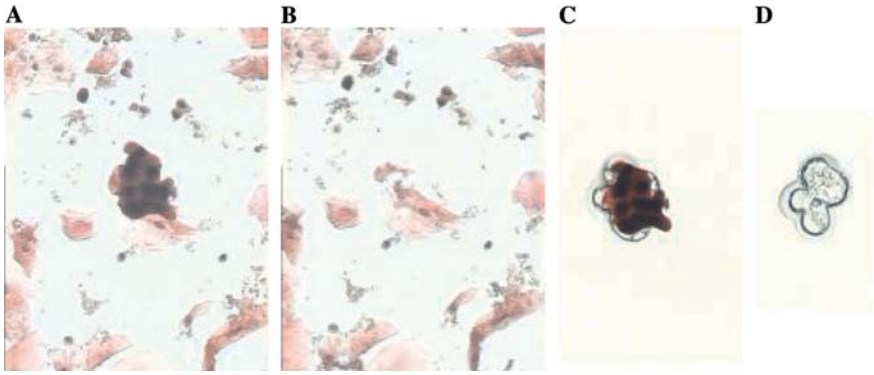

Fig. 1. Example of laser capture of epithelial cells from a sputum sample. Sputum cells were collected on a membrane by sputum cytocentrifugation. (A) Collected cells were stained with hematoxylin and eosin and then histopathologically analyzed. Malignant epithelial cells appeared as a group of dark-stained cells. Nonmalignant epithelial cells, buccal epithelial cells, and inflammatory cells can be also observed. Nonmalignant epithelial cells and buccal cells were also taken on separate caps for mutation analysis and for comparison with malignant epithelial cells. (B) Cells in A after the cluster of malignant epithelial cells has been taken. Malignant cells captured on a cap (C) were lysed to release the cellular material. (D) Remainder of captured cells after treatment with proteinase K.

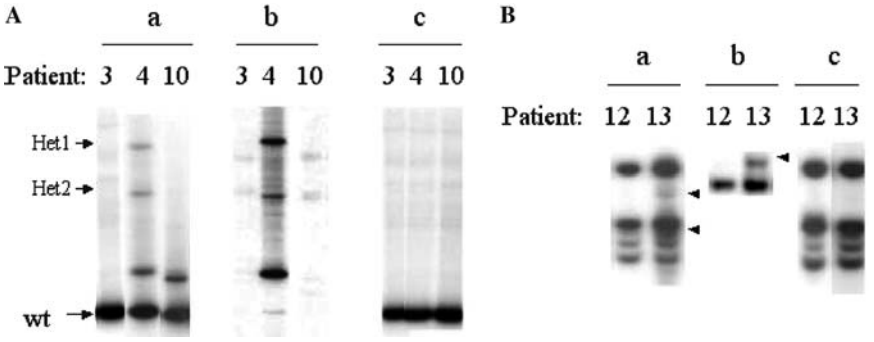

Fig. 2. Molecular analysis of sputum samples of lung cancer patients. Example of mutation analysis for the K-ras gene (A) for three patients (patients 3, 4, and 10) and for the p53 gene (B) for two patients (patients 12 and 13). For each patient, mutations were analyzed by three approaches. First, epithelial cells were taken by laser capture microdissection from each sputum and analyzed for K-ras mutations by PCR + DGGE (a in A) and for p53 mutations by PCR + SSCP (a in B). Second, DNA was extracted directly from a fraction of each sputum. In A, an aliquot of the DNA was analyzed for K-ras mutations by PCR + MAE + DGGE (b) and by PCR + DGGE (c). In B, an aliquot of the DNA was analyzed for p53 mutations by PCR + MAE + PAGE (b) and PCR + SSCP (c). In A, wt indicates the position of wild-type K-ras exon 1 allele in the gel. For patient 4, a mutant pattern appears (a4 and b4) and corresponds to the two respective mutant/wild-type heteroduplexes (het1 and het2) and the mutant homoduplex focusing between the wt and the het2. In B, arrowheads indicate the positions of p53 mutant DNA fragments appearing in sputum of patient 13 (a13 and b13).

cytocentrifugation and laser capture microdissection. The captured cells were then used for screening for K-ras mutations using PCR + DGGE and for p53 mutations using PCR + SSCP. Fig. 1 shows a group of malignant cells collected on a filter membrane by cytocentrifugation of a sputum sample obtained from one of the patients before (A) and after (B) laser capture. Sputum cells consisted of a mixture of mostly leucocytes, buccal cells, and malignant and/or atypical epithelial cells. About 10% of sputum cells from this patient corresponded to malignant and atypical epithelial cells. Approximately 100 malignant cells in each sputum sample were laser-captured on a cap and molecularly analyzed. The cells captured on a cap (C) were then lysed to release the cell content used for mutation analysis. D shows the captured cells after treatment with proteinase K.

Fig. 2 shows an example of mutation analysis, in A by DGGE in exon 1 of the K-ras gene for 3 patients (patients 3, 4, and 10) and in B by SSCP and PAGE in exons 5–8 of the p53 gene for 2 patients (patients 12 and 13). In A, the analysis was performed using cells taken from sputum and the PCR + DGGE method (a), or DNA extracted from sputum and the PCR + MAE + DGGE method (b), or the PCR + DGGE method (c). DGGE analysis showed that patient 4 revealed K-ras codon exon 1 mutant sequences in the laser-captured cells (indicated by het1 and het2 in lane a4). This mutant corresponded to a GGT to TGT mutation in codon 12 of the K-ras gene. This same mutation was also detected in DNA isolated from sputum cells of this patient by the PCR + MAE + DGGE method (lane b4), but not by the PCR + DGGE method (lane c4). For comparison,

patient 10 did not reveal any mutant sequence pattern when DNA extracted from sputum cells was analyzed by using either the PCR + DGGE (lane c10) or the PCR + MAE + DGGE (lane b10) method. However, when cells isolated from this patient's sputum were analyzed by even the PCR + DGGE method a mutant sequence corresponding to a GGC to TGC mutation in codon 13 of the K-ras gene was detected (lane a10). As expected, this mutation was not detected using the PCR + MAE + DGGE method because it targeted only codon 12 mutations (lane b10). For comparison, patient 3 showed no mutations in either the cells isolated from sputum (lane a3) or the DNA extracted from sputum cells by using both the PCR + MAE + DGGE (lane b3) and the PCR + DGGE (lane c3) methods.

In B, p53 mutations were screened by using cells isolated from sputum and the PCR + SSCP method in a, DNA extracted from sputum and the PCR + SSCP method in c, or the PCR + MAE + PAGE method in b. Patient 13 showed a p53 mutant in cells captured from sputum (lane a13). This mutant corresponded to a CGG to CAG mutation at codon 248 of the p53 gene. The identical mutation was detected in DNA extracted from sputum by using the PCR + MAE + PAGE method (lane b13), but not by using directly the less sensitive PCR + SSCP method (lane c13).

Table 1 summarizes the mutations detected using three approaches in sputum samples of 7 (46.6%) of the 15 patients investigated. Five patients had each a p53 mutation, including patient 2 (with a silent CAA to CAG mutation at codon 136 in exon 5), patient 7 (with a CCC to TCC mutation at codon 151 in exon 5), patient 8 (with a GGC to TGC mutation at codon 244 in exon 7), patient 13 (with a CGG to CAG mutation at codon 248 in exon 7), and patient 14 (with a GGC to GTC mutation at codon 244 in exon 7). Patient 4 had a GGT to TGT mutation in codon 12 of the K-ras gene. Patient 10 had a GGC to TGC mutation at codon 13 of the K-ras gene and an AAG to TAG mutation at codon 139 in exon 5. None of these mutations were detected in the matched nonmalignant epithelial cells or the matched buccal cells taken from sputum of these patients (data not shown). Therefore, only two mutations were detected in DNA extracted directly from sputum of two patients, including a K-ras codon 12 mutation (patient 4) and a p53 mutation at codon 248 (patient 13), by using sensitive methods. Both mutations and six additional mutations, including a K-ras codon 13 mutation and five p53 mutations, were detected when only epithelial cells isolated from sputum were analyzed by using less sensitive but less laborious methods.

Taken together, these results showed that p53 and K-ras mutations were frequent in sputum samples of lung cancer patients from Xuan Wei County, China. These mutations were detected in only malignant epithelial cells. Most of them occurred outside the hotspot codons and therefore were not detected by the sensitive method targeting only mutations at hotspot codons. Although the number of patients analyzed in this study was small, the observed frequency of p53 mutations (46.6%) is in agreement with those reported by previous studies of p53 mutations and K-ras mutations in lung tumors obtained from lung cancer patients from Xuan Wei County [41,42]. These results demonstrate the usefulness of the combination of epithelial cell isolation and mutation analysis as a sensitive screening approach for p53 mutations in sputum samples of lung cancer patients.

Both K-ras and p53 mutations were detected in 30–50% [8–12] of all non-small-cell lung carcinomas [8–19]. Furthermore, these mutations were reported to define independent prognostic markers for lung cancer [9,10,23]. Therefore, the high prevalence of these two gene mutations combined in lung cancer suggests that they provide useful biomarkers for lung cancer. Several studies also showed that both K-ras and p53 mutations were detected in sputum and BAL samples obtained from lung cancer patients, using sensitive codon-specific methods. In some studies, these mutations were detected in archival sputum samples obtained from patients 6 months to 1 year prior to observable clinical symptoms [30,32,35]. Taken together, these results suggest that sensitive detection of these mutations in sputum or BAL samples of individuals with a high risk for lung cancer could provide a useful approach for early detection of this disease.

In summary, we show that the combination of laser capture microdissection and SSCP/DGGE facilitates analysis of low-fraction mutations occurring in exons of the p53 and K-ras genes in sputum of lung cancer patients. This approach should be also useful for investigation of mutations in these genes or any other gene relevant to lung cancer in sputum or BAL samples of lung cancer patients and individuals with a high risk for lung cancer.

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